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# Phylogenetic Position of *Geosmithia* spp. (Hypocreales) Living in *Juniperus* spp. Forests (Cupressaceae) with Bark Beetles of *Phloeosinus* spp. (Scolytinae) from the Northeast of Mexico

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**Abstract:** *Geosmithia* members are mitosporic filamentous fungi commonly recorded and isolated from bark beetles of the Scolytinae subfamily and their respective host's species. This genus includes 18 species formally described and 38 phylogenetic species recorded in several localities from Africa, Asia, Australia, Europe, and North and South America, where they exhibit frequent associations with phloeophagous and wood-boring bark beetles. Among phloephagous bark beetle species, specifically, in members of the genus *Phloeosinus* Chapuis, almost 10% of *Geosmithia* strains have been isolated. By its physiographic elements and high bark beetle and conifer species richness, Mexico is a potential region to host a high diversity of *Geosmithia* species and potential new species. In the present study, we systematically sampled and isolated, cultured, and molecularly identified members of the *Geosmithia* species associated with *Phloeosinus* spp. and their *Juniperus* spp. host trees at the north of Sierra Madre Oriental, at Nuevo Leon State, Mexico. Phylogenetic analyses based on 378 internal transcribed spacer region (ITS) sequences supported the presence of strains from *Geosmithia langdonii-Geosmithia* sp. 32 clade associated with *Phloeosinus serratus* vector and with *Juniperus coahuilensis* (JC) host, and the presence of strains from *Geosmithia* sp. 21-*Geosmithia xerotolerans* clade with *Phloeosinus deleoni* and *Juniperus flaccida* (JF) in this geographical region. The genetic and morphological differences found in our strains with respect to those previously described in the species from both clades (*Geosmithia langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*G. xerotolerans*) suggest that both *Geosmithia* lineages from Nuevo Leon correspond to two potential new species in the genus.

**Keywords:** bark beetles; *Geosmithia*; *Phloeosinus*; *Juniperus*

## 1. Introduction

Associations among fungi and bark beetles constitute one of the most successful ecological adaptations that promoted complex and dynamic interactions in this insect group [1]. Most of these

associations are driven by the host tissue within which the beetles develop. Many fungal species are saprophytes on wood and inner bark, but others are nutritional mutualists and facultative or obligate components of wood-boring insects' diet [2].

Some bark beetles maintain obligatory functional and physiological dependent associations with filamentous fungi [3]. These insects actively cultivate the fungi within the gallery tunnels, constituting agricultural systems that provide a source of food to both larvae and adults, and in some cases, hormones associated with the molting and metamorphosis processes [4]. Bark beetles with facultative associations do not cultivate the fungi and do not need them to complete their life cycle, although in some species they can enrich their diet and increase their fitness [5].

The common fungal species associated with bark beetles belong to eight genera from Basidiomycota (*Phlebiopsis* Jülich, *Entomocorticium* Whitney, Bandoni & Oberw) and Ascomycota (*Ophiostoma* Syd & Syd, *Grosmannia* Goid, *Ceratocystis* Ellis & Halst and *Leptographium* Lagerb & Melin, *Raffaelea* Arx & Hennebert, *Graphilbum* Upadhyay & Kendr) [6–8]. Most of them are from Ophiostomatales and Microascales orders, which can be actively cultivated as the sole source of food by almost 30% of bark beetle species [9,10]. However, many other non-incidental fungal species have been recorded in insect galleries and are often understudied, such as species of genus *Geosmithia* Pitt (Ascomycota: Hypocreales) [10–12].

*Geosmithia* members are mitosporic filamentous fungi, characterized by macronematous conidiophores that produce large chains of conidia and hydrophobic and dry spores [13]. The current diversity of this genus includes 18 species formally described and 38 phylogenetic species named numerically without a formal taxonomic description [13–25]. These species are widely distributed with records from Africa, Asia, Australia, Europe, and North and South America [16,19,23,24], where they exhibit various degrees of specificity with their hosts. Some species are collected from subcortical insects, and other species are sporadically isolated from other substrates such plant debris, soil, and cereals [13,26,27]. While *Geosmithia* spp. can provide the main nutritional source for their vectors, little is known about the interactions of other symbiotic *Geosmithia* species with bark beetles. In particular, their role as pathogens remains undetermined [17]. Currently, only *Geosmithia morbida* Kolarık, Freeland, Utley & Tisserat dispersed by the walnut twig beetle *Pityophtorus juglandis* Blackman is considered a phytopathogen, which is a serious threat to black walnut trees (*Juglans nigra* Linnaeus) as it causes thousand cankers disease [17].

Several members of *Geosmithia* have been recorded and isolated with higher frequency from Scolytinae than from other groups of beetles, which corresponds to 30 genera of bark beetles [11,13–16,19–21,28]. The most diverse group of *Geosmithia* species is associated with beetle vectors that feed on conifer trees [10,11]. Typically, *Geosmithia* is found in association with phloephagous and wood-boring bark beetles and their respective host plants [13–15]. Among phloephagous bark beetle species, within members of the genus *Phloeosinus* Chapuis, almost of 10% of all *Geosmithia* strains known have been isolated [16,19,29].

*Phloeosinus* Chapuis (Curculionidae, Scolytinae) is a medium–large bark beetle genus, which includes about 80 species spread among all continents [30–33]. Most of these species breed with members from Cupressaceae (*Cupressus* sp., *Chamaecyparis* sp., *Thuja* sp., *Juniperus* sp.) [30,34]. The life cycle of *Phloesinus* species involves feeding and reproduction into the phloem of recently dead trees (branches and trunks), dying trees, or weakened trees [35]. As a consequence of the construction of their galleries, the attacked trees can lose their ornamental appearance and eventually die; nonetheless, when the population growth of these beetles is high, some species produce considerable tree mortality. The ecological role of these insects is to promote the regeneration of natural forests, although in some cases are considered urban pests from an anthropocentric point of view [36].

There are at least 30 reports in the world about the *Geosmithia* associated with bark beetles (e.g., *G. langdonii-Scolytus intricatus*; *G. proliferans-Phloeotribus frontalis*; *G. brunnea-Xylosandrus compactus*; *G. morbida-Pityophthorus juglandis*, etc. [19,29]); however, little is known about the overall diversity and vector spectrum of this fungal genus in North America. Current studies indicate that the genus

in this region is highly diverse based on extensive systematic samplings from several "vector" bark beetles and host plant species. Numerous valid documented and undescribed phylogenetic Geosmithia species have been discovered through sampling from Western and Southeastern USA [19,29].

Species discovery is still a major endeavor of the field of taxonomy. In some taxa, it is calculated that approximately half of the new species are discovered from samplings of only a few specimens and localities. Despite the fact that this practice provides incomplete distribution and morphological data, species discovery from a few specimens/localities provides the necessary information to help taxonomists know it and relative taxa [37].

Because of its physiographic elements and high bark beetle and conifer species richness, Mexico is a region expected to host a high diversity of *Geosmithia* and potential new fungal species; in spite of this, there are no records of these symbiotic associations in the country. Therefore, we conducted a survey to study bark beetles of the genus *Phloeosinus* and its host plants in Nuevo Leon state, center of Sierra Madre Oriental (SMOr) Mexico, to explore *Geosmithia* diversity and determine its possible association with-bark beetles and their plant hosts.

The Sierra Madre Oriental (SMOr) is a mountain system considered a biotic unit in different regionalization proposals [38]. Different biomes are distributed within it, which in turn are home to a high level of biological diversity [39,40]. At least three *Phloeosinus* species inhabit the north of this region, *P. baumanni* Hopkins, *P. deleoni* Blackman, and *P. tacubayae* Hopkins, two of them endemic to the country (https://www.barkbeetles.info/index.php). Seven *Juniperus* species are also distributed, namely *J. angosturana* R. P. Adams, *J. coahuilensis* (Martínez) H. Gaussen ex R. P. Adams, *J. depeanna* Steud, *J. flaccida* Schltdl, *J. pinchotii* Sudw, *J. saltillensis* T. M. Hall, and *J. zanonni.* R. P. Adams [40], on host species that have previously been demonstrated to harbor a high frequency of *Geosmithia* species in other latitudes [19,30]. This ratifies the importance of studying *Geosmithia* diversity in Mexico.

The goal of the present study is to explore the diversity of *Geosmithia* associated with *Phloeosinus* bark beetles with Juniper host preferences in the north of the SMOr, at Nuevo León State, Mexico. Through isolation, culture, morphological and molecular techniques, we associate fungal strains with the recognized phylogenetic groups in the genus and compare morphological attributes of isolated strains with those shown in the described species, to evaluate the presence of potential species in *Geosmithia* in this unexplored region.

## 2. Materials and Methods

### 2.1. Sampling

Potential vector bark beetles and their tree hosts were collected from June to December 2019 from two areas from Nuevo Leon State, SMO, located in the northeast of Mexico (Figure 1). One of them is located 27 km northwest of the municipality of Galeana, 400 m from kilometer 145 of the Matehuala-Monterrey highway, in an area of undisturbed open vegetation, with semi-arid xerophytic scrub dominated by the *Juniperus coahuilensis* (JC)species in an arboreal state (Figure 2a,b), while the second one is 4 km away from Iturbide municipality, 100 m from the Iturbide-school forest of the Universidad Autónoma de Nuevo León highway, in an area of semi-arid pine forest and transition of between *Pinus* species and *Juniperus flaccida* (JF), where the latter dominates in an arboreal state (Figure 2h,i).

In each area, healthy trees of the Juniper species were selected, and we deployed freshly cut branches of the targeted tree as a lure for bark beetles. The cut branches were 80–100 cm long × 10–15 cm diameter and were laid on the floor near to the tree they were obtained from, for environmental exposure for approximately 1–2 months (Figure 2c,i). The branches were monitored weekly to assess the occurrence of colonizing beetles, which can be recognized by the presence of a sawdust-like substance, called frass, created by bark beetles colonizing, which is accumulated in tree crevices and may have fallen to the floor gallery, resembling very fine, cream-brown coffee ground material at the floor, together with branches (Figure 2d,e,k,l). Those branches with colonization signals were collected and

transferred to the laboratory of Entomology of Facultad de Ciencias Forestales, Universidad Nacional Autónoma de Nuevo León, Linares Nuevo León state for its protection and examination. In total, 11 cut branches were sampled, 4 of them corresponding to *J. coahuilensis* from Galeana municipality and the remainder to *J. flaccida* from Iturbide. Sampling is displayed in Table 1.

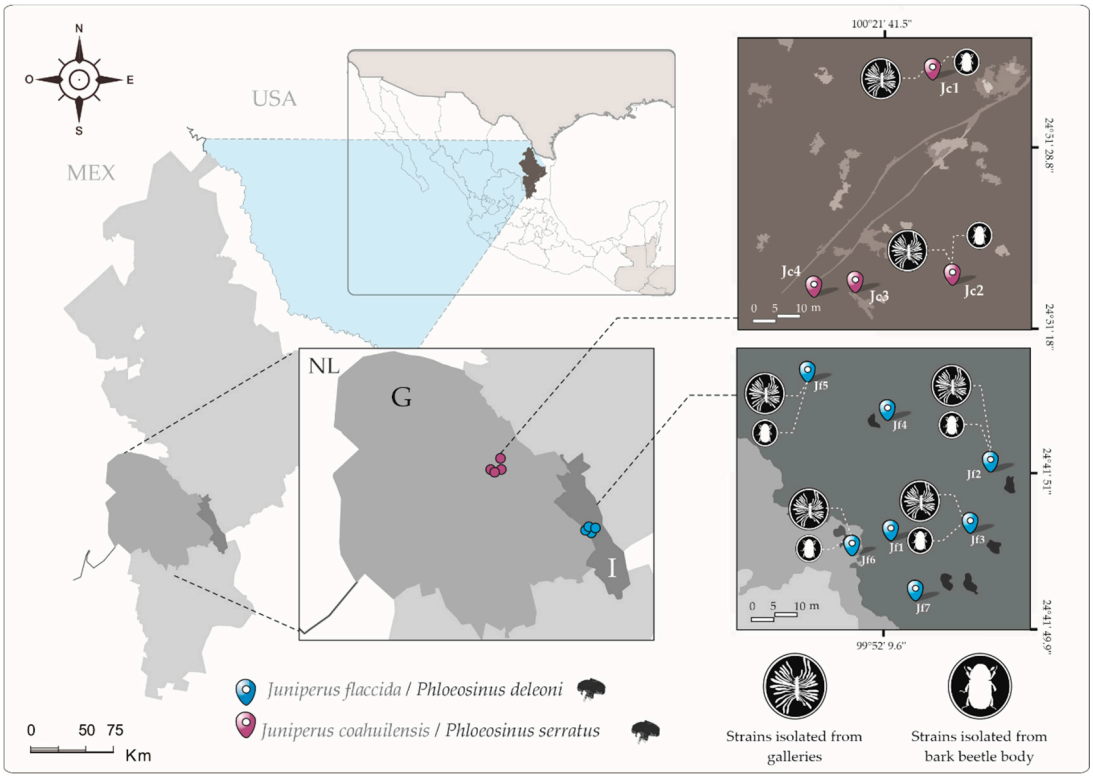


**Figure 1.** Map of sampling sites of *Geosmithia* spp. in Nuevo León, México. Acronyms of samples in Table 1.

**Table 1.** Sample acronym of freshly cut branches of Juniper species, locality, host species, and bark beetle species vector, studied in Nuevo León State, Mexico.

| Acronym | Locality | Longitude W | Latitude N | Host | Vector |
|---|---|---|---|---|---|
| JC1 | Area one, Galeana | 24°51′34.8″ | −100°21′37.2″ | *Juniperus coahuilensis* | *Phloeosinus serratus* |
| JC2 | | 24°51′17.9″ | −100°21′46.0″ | | |
| JC3 | | 24°51′18.3″ | −100°21′36.0″ | | |
| JC4 | | 24°51′18.1″ | −100°21′42.9″ | | |
| JF1 | Area two, Iturbide | 24°41′50.2″ | −99°52′8.9″ | *Juniperus flaccida* | *Phloeosinus deleoni* |
| JF2 | | 24°41′50.5″ | −99°52′9.5″ | | |
| JF3 | | 24°41′50.4″ | −99°52′9.8″ | | |
| JF4 | | 24°41′51.5″ | −99°52′9.6″ | | |
| JF5 | | 24°41′51.8″ | −99°52′10.2″ | | |
| JF6 | | 24°41′50.0″ | −99°52′9.3″ | | |
| JF7 | | 24°10′4.9″ | −100°4′5.6″ | | |

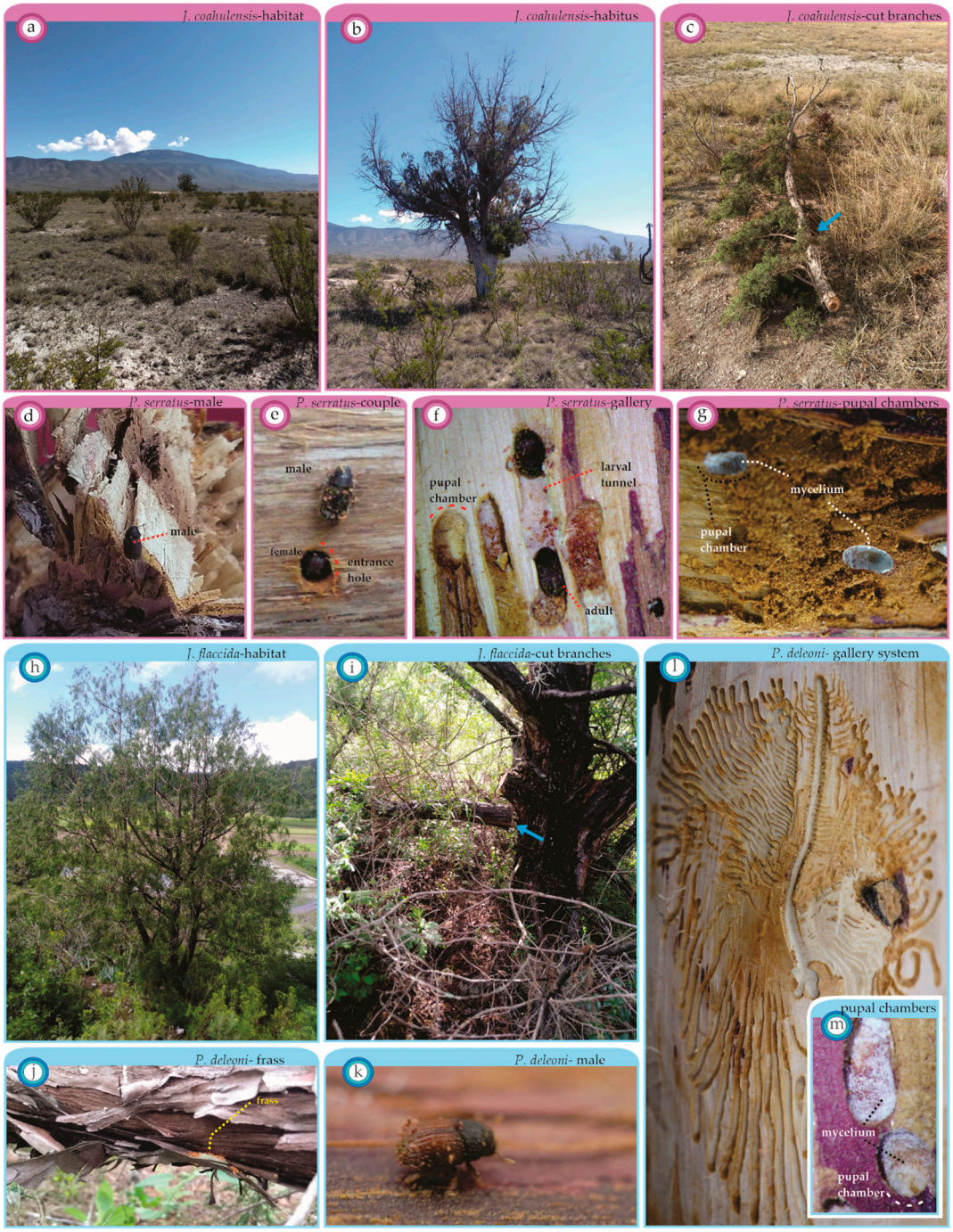


**Figure 2.** A sampling of the *Juniperus* hosts and *Phloeosinus* vector species studied. Pink and blue squares correspond to samples from Galeana (**a**–**g**) and Iturbide (**h**–**m**) municipalities, respectively, Nuevo Leon, Mexico. (**a**) Semi-arid xerophytic scrub landscape; (**b**) habitus of *Juniperus coahuilensis;* (**c**) cut branch of *J. coahuilensis* used as a lure; (**d**) male of *Phloeosinus serratus* on bark; (**e**) male and female of *P. serratus* in entrance hole; (**f**,**g**) larval galleries and pupal chambers of *P. serratus* with mycelium of strains from *Geosmithia langdonii-Geosmithia* sp. 32 clade; (**h**) habitus of *J. flaccida* in semi-arid forest; (**i**) cut branch of *J. flaccida* used as a lure; (**j**) frass as a sign of bark beetles colonizing; (**k**) male of *P. deleoni* on bark; (**l**,**m**) the gallery system and pupal chamber of *P. deleoni* with a signal of growth of mycelium of strains from *Geosmithia* sp. 21.-*G. xerotolerans* clade.

Trunks were debarked to expose the wood, galleries, and beetles (Figure 2f,j). The bark was removed in both the vertical plane and the entire circumference. In each gallery system, some bark

beetles were removed with tweezers, stored in 70% alcohol for identification and in Petri dishes for fungal isolation, without mixing insects from different gallery systems. Bark beetle adults were identified by external morphological characters using the taxonomic key of Wood [31].

The isolation of putative *Geosmithia* spp. was realized directly from gallery systems and bark beetle adults of the *Phloeosinus* species. Of each trunk colonized, at least one gallery system and the respective insects from it were sampled. The fungus was scraped from the gallery surface if growth of mycelium was observed on it (Figure 2g,m). For the bark beetles, the collection was done by vortexing a pull of whole beetles (10 specimens) in a 1.5-mL tube containing 1 mL of sterile wash solution (0.02% Tween 80 solution in water) for 1 min. The fungal scraping and 100 mL of wash solution of insect bodies were inoculated onto Petri dishes with Malt Extract Agar (MEA2, BD Difco) Czapek yeast autolysate agar (CYA) [23] supplemented with trace elements (0.001% $ZnSO_4$-$7H_2O$ and 0.0005% $CuSO_4$-$5H_2O$, and Panela Medium Agar (PMA) [41]. Parafilm-sealed Petri dishes were inverted in plastic containers, incubated in the dark at 28 °C, and examined daily for 14 days.

### 2.2. Cultural and Morphological Characteristics

The identification and morphological characterization of the *Geosmithia* isolated followed the protocol of Pitt [23]. Pure cultures of *Geosmithia* spp. were obtained by using a sterilized mycology handle to take some sample and reseed in other MEA2, CYA, and PMA plates, which were incubated at 25 °C for 7–14 d with an examination at 24 h intervals until the emergence of *Geosmithia* fruiting structures. Additionally, a duplicate slide culture with CYA media was realized to observe the reproductive structures of the *Geosmithia* as described Harris [42].

To observe the reproduction structures with scanning electron microscopy (SEM) and phase-contrast microscopy (PCM), a slide culture technique for fungi was performed following the techniques described by Aylmore and Todd [43]. For each *Geosmithia* culture, three slides were prepared, one of them for PCM and the remainder for SEM. In brief, square blocks (5 mm per side) of the CYA medium were cut; blocks on the slides were inoculated on four sides of the CYA square with mycelial fragments; an agar cube was covered with a coverslip on the upper surface and incubated for 48 h. The cover glass was removed from the slide culture when hyphae and production of spores were observed over the surface of the glass. Fungal structures were observed using a lactophenol cotton blue stain on a clean microscope slide. For electronic microscopy, the glasses with hyphae and spores adhered were dehydrated and critical point dried with $CO_2$, mounted, and coated with a mixture of gold-palladium and subsequently observed by SEM in a Hitachi model SUI510 scanning electron microscope.

Conidiophore and ontogenesis of conidia were observed in plate cultures according to Cole et al. [44] and incubated in daylight for the best development of conidiophore roughness. Micromorphology was studied on seven-day-old colonies grown on MEA and CYA, and the conidiophores were taken from margins and near colony centers, as well as from areas that differed in their texture. A total of 20 randomly selected conidia of each strain were measured. The substrate mycelium from the colony margins was studied for the presence of substrate conidia. Mounts were prepared in Melzer's reagent and 20% lactic acid with 0.05 g cotton blue.

### 2.3. DNA Extraction, Amplification, and Sequencing

Genomic DNA of *Geosmithia* isolates was extracted from pure cultures by following the protocol of Hernandez-García et al. [45]—the DNA genomic was stored at −20 °C until use. Extractions were performed from pure isolated fungi coming from each debarked gallery system, corresponding to both isolates from scraped galleries and insects contained in they ("wash solution of insect bodies"). A region that ranged from 300 to 500 bp for the internal transcribed spacer region (ITS) was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [46]. The PCR amplifications were performed in a TC-5000 thermocycler (Techne, Staffordshire, UK) using a total reaction volume of 25 µL, which contained 50–100 ng DNA template, 1X reaction buffer,

2.0 mM $MgCl_2$, 0.4 µM each primer, 0.4 mM dNTPs, and 1 U Taq polymerase (Invitrogen Life Technologies, Sao Paulo, BR). The reaction conditions were the following: initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplification products were purified and sequenced in the Laboratory of Genomic Sequencing of Biodiversity and Health, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico.

### 2.4. Phylogenetic Analyses

Taxonomic identification of the *Geosmithia* isolates was based on the similarity level with respect to reference sequences from the GenBank [13–25]). The alignment was carried out with the program ClustalW [47] and sequences were edited using the program Seaview [48]. The sequences were deposited in GenBank under the accession numbers (MT969332-MT969343).

To evaluate the phylogenetic position of the obtained *Geosmithia* sequences with respect to *Geosmithia* spp. previously associated with bark beetles, a series of phylogenetic analyses (PA) by Maximum likelihood (ML) were performed. Thus, 366 sequences of the ITSs corresponding to 18 species formally described and 38 undescribed phylogenetic species of *Geosmithia* spp. available in public databases from several studies were used in our analyses [15,19,21,25,29,49]. Each sequence was considered a molecular operational taxonomic unity (MOTU). In the first phylogenetic analysis (PA1) to estimate the relationship of target sequences concerning the "five" *Geosmithia* complexes previously recognized [11], a data set of 378 *Geosmithia* sequences was included, to *Acremonium alternatum* Link (AY566992.1) and *Emericellopsis pallida* Beliakova (NR_145052.1) as outgroups. Subsequent phylogenies were reconstructed to locate *Geosmithia* strains isolated within the complexes. In these analyses, only the sequences from the closest clades to the target sequences displayed in PA1 were included, the most distant MOTU's with respect to the most inclusive monophyletic clade were used as an outgroup.

The best nucleotide substitution model for each analysis was determined in jModelTest 2.1.10 [50] and selected based on the lowest Akaike Information Criterion (AIC) value. Maximum likelihood (ML) phylogenetic analyses were conducted by using IQTREE v 1.6.12 [51] with recommended partition parameters. To assess the tree topology, Bootstrap support in IQTree was calculated using the ultrafast option [52]. The tree was visualized and edited by using FigTree 1.4.4 [53], and modified using Inkscape (https://inkscape.org/en/). The genetic distances of Nei, between and within target *Gesomithia* sequences, and for the closest, MOTUs were calculated in MEGA 10.1 [54].

## 3. Results

Bark beetles were attracted to six of 11 cut branches used as lures and traps, two belonging to *J. coahuilensis* from the Galeana municipality and four belonging to *J. flaccida* from the Iturbide municipality (Figure 1). Based on morphological attributes, two species of *Phloeosinus* were identified on these hosts, *Phloeosinus deleoni* Blackman in *J. flaccida*, and *P. serratus* in *J. coahuilensis*. From these samples, 12 gallery systems (gs) and their respective bark beetles were studied; four gs of *P. serratus* and eight of *P. deleoni*. In all gallery systems of both species, the growth of mycelium was observed as a thin layer of withe velvety powder covering the walls of the gallery system, which was conspicuously evident on the pupal chambers of the gallery systems (Figure 2g,m).

The fungal isolation was performed directly from 12 gs and 12 insect pulls of their respective bark beetle adults; from these, 24 pure cultures were obtained (gs = 12; insects = 12), which, utilizing cultural and micro-morphological traits [13–15,26], were classified into two morphs, one of them isolated from *J. flaccida-P. deleoni* (gs = 8; insects = 8) and the other one from *J. coahuilensis-P. serratus* (*gs* = 4; insects = 4).

Presumptive molecular identification of the 24 isolates based on ITS sequences using blastn NCBI (https://blast.ncbi.nlm.nih.gov/), supported that all obtained sequences correspond to *Geosmithia* genus. Sequences of isolates from *J. flaccida-P. deleoni* (gs = 8; insects = 8) showed around 95.7% identity and 99% of query coverage with *Geosmithia* sp. CCF3355 isolated of *Phloeosinus punctatus* LeConte from *Juniperus occidentalis* Hook. Sequences isolated from *J. coahuilensis-P. serratus* (gs = 4;

insects = 4) showed around 98.7% identity and 98% of query coverage with *Geosmithia langdonii* U91 associated to *Bostrichidae* sp. from host feeding on *Baccharis pilularis* DC, both reported in California, USA. Sequences edition and alignment show that from the 12 *Geosmithia* sequences associated with *J. flaccida-P. deleoni* and from eight recover from *J. coahuilensis-P. serratus* that six and two corresponded to similar haplotypes, respectively; they were not included in the phylogenetic analysis. For PA1, the aligned ITS dataset included 366 sequences plus 12 target sequences around 500 bp (n = 378).

### *3.1. Phylogenetic Analysis*

The first maximum likelihood phylogenetic analysis (PA1) based in six *Geosmithia* sequences isolated from *J. flaccida-P. deleoni,* six from *J. coahuilensis-P. serratus* and including 378 ITS sequences of 18 species formally described and 38 undescribed species of *Geosmithia*, recovered two big clades previously shown in others studies ([11,19,29]; Figure 3a). Group 1 is composed mainly by, *G. pallida* Kolarik, Kubatova and Pazoutova, *G. carolliae* Cunha, Machado & Souza-Motta, *Geosmithia* sp. 2, and unclassified *Geosmithia* species; Group 2 is composed by *G. cnesini* Kolařík Kirkendall, *G. omnicola* Pepori, Kolařík, Bettini, Vettraino and Santini, *G. ulamcea* Pepori, Kolařík, Bettini, Vettraino and Santini, *G. eupagioceri* Kolařík, *G. langdonii* Kolařík, Kubátová and Pažoutová, *G. flava* Kolařík, Kubátová and Pažoutová, *G. morbida* Kolařík, Freeland, Utley and Tissera, *G, fasssatiae* Kolařík, Kubátová and Pažoutová, *G. microcorhtyli* Kolařík, *G. rufescens* Kolařík, *G. lavendula* Pitt, *G. puterillii* Pitt, *G. obscura* Kolařík, Kubátová and Pažoutová, and unclassified, including *Geomsithia* sp. 21 (Figure 3b). In these clades, the "five" well-defined groups corresponding to the *Geosmithia* complexes were observed as previously recognized [11]. All target sequences from *J. flaccida-P. deleoni,* were located with a bootstrap value of 98% within a clade integrated mostly by the sequences of *Geosmithia* sp. 21 and the only available sequence of the recently described specie *G. xerotolerans* [25]. All *Gesomithia* sequences from *J. coahuilensis-P. serratus* were included within the clade integrated by of *Geosmithia langdonii* Kolarik, Kubatova, Pazoutova and *Geosmithia* sp. 32 with a bootstrap value of 67% (Figure 3b).

Two subsequent phylogenetic trees were estimated, one of them focused on clarifying the position of sequences associated in the clade of *G. langdonii-Geosmithia* sp. 32, and the other one on those included in the *Geosmithia* sp. 21-*G. xerotolerans* clade. In both analyses, the target sequences were recovered within monophyletic groups, respectively, with bootstrap values of 100%. Phylogeny of *G. langdonii-Geosmithia* sp. 32 clade associated the sequences isolated from *J. coahuilensis-P. serratus* with *G. langdonii* obtained from *Thuja occidentalis* L. three host, and associated with *Phloeosinus thujae* Chapuis beetle vector from California, USA [HF546250.1; 19] with 96% bootstrap (Figure 4); average genetic distances of target sequences with respect to other *G. langdonii-Geosmithia* sp. 32 were 1.9%–3.1%, ($x$ = 2.6%). The phylogeny of clade *Geosmithia* sp. 21-*G. xerotolerans*, associate the sequences isolated from *J. flaccida–P. deleoni* with *Geosmithia* sp. 21 isolates obtained from *Ficus carica* L. three host, and associated with *Hypoborus ficus* Erichson bark beetle vector from Aquitánie, France, reported by Kolarik et al., [15] with a 73% of bootstrap (Figure 5), average genetic distances of target sequences with respect to other *Geosmithia* sp. 21-*G. xerotolerans* were 3.7%–5.1% ($x$ = 4.7%).

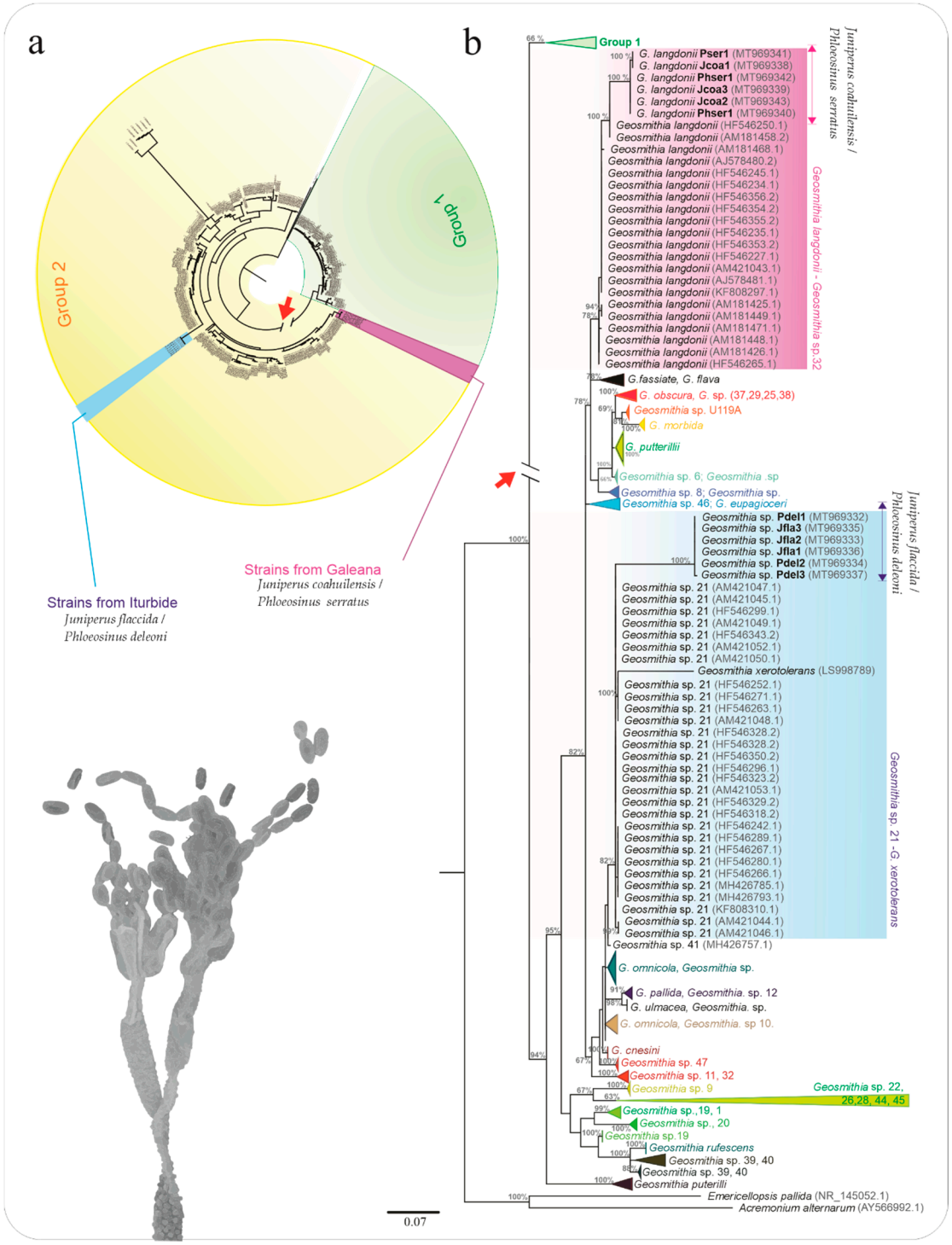


**Figure 3.** Phylogenetic relationship of the *Geosmithia* spp. isolated based on the internal transcribed spacer region (ITS). Pink and blue colors correspond to *Geosmithia langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*G. xerotolerans* clades, respectively. (**a**) The phylogenetic tree resulting from the Maximum likelihood (ML) analysis of 378 ITS sequences; (**b**) "Group 2" of the phylogeny displayed in "a". Red arrows indicate the cutting point. Target sequences of *Geosmithia* of this study are shown in bold. *Emericellopsis pallida* and *Acremonium alternarum* were selected as outgroups.

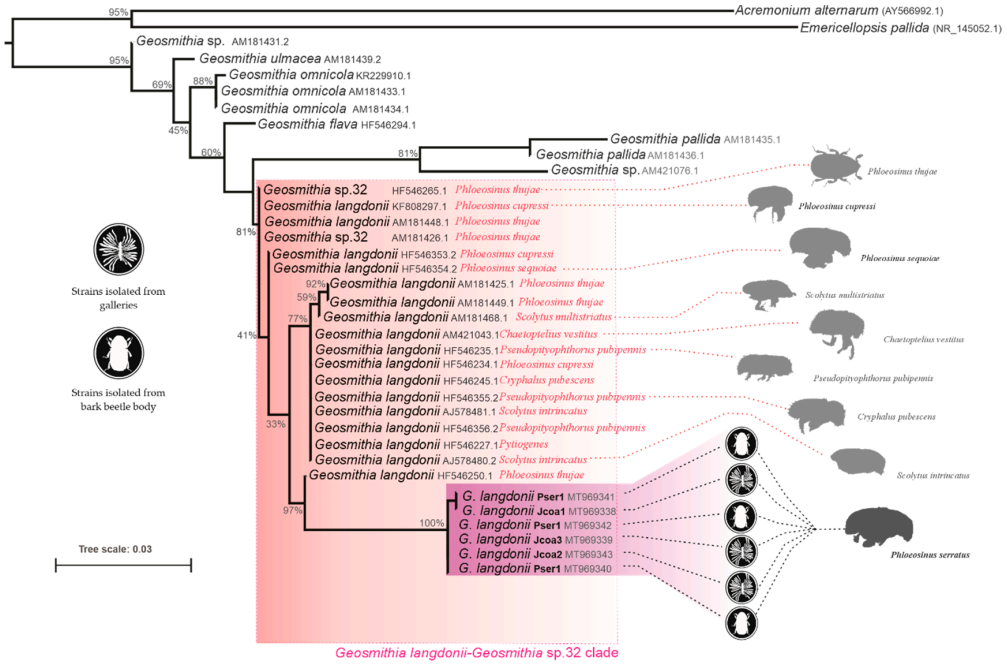


**Figure 4.** Phylogenetic relationships among strains from *Geosmithia langdonii-Gesomithia* sp.32 from Galeana, Nuevo León and its most closely related species based on the ITS sequences. The phylogenetic tree was obtained by ML analysis of 34 ITS sequences of *Geosmithia langdonii* and *Geosmithia* sp. 32. *Emericellopsis pallida* and *Acremonium alternarum* were selected as outgroups. Acronyms Pser and Jcoa correspond to strains isolated from *Phloeosinus serratus* and *Juniperus coahuilensis*, respectively.

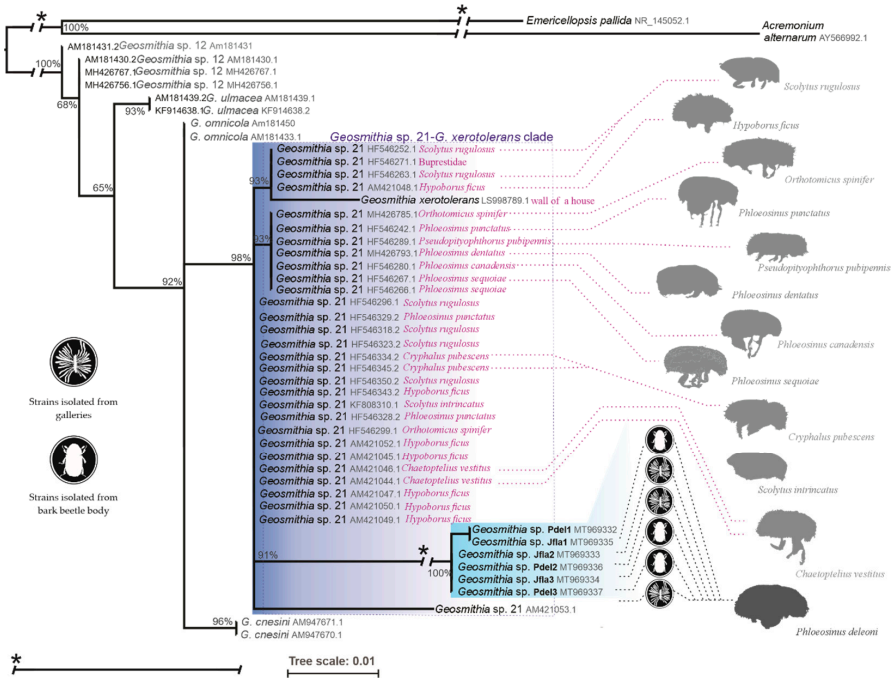


**Figure 5.** Phylogenetic relationships within of strains from *Geosmithia* sp. 21-*G. xerotolerans* clade from Galeana, Nuevo León and its most closely related species based on the ITS sequences. The phylogenetic tree was obtained by Maximum Likelihood from 49 *Geosmithia* sequences. *Emericellopsis pallida* and *Acremonium alternarum* were selected as outgroups. Acronyms Pdel and Jfla corresponding to strains isolated from *Phloeosinus deleoni* and *Juniperus flaccida*, respectively. The asterisks on the tree indicate the sections of the branches that were cut out, for representational purposes. The straight line with the asterisk under the tree indicates the length of the section that was cut, which was the same in all cases.

### 3.2. Morphological Characterization

Strains obtained from *J. flaccida-P. deleoni* (Figure 6a–f). Conidiophores on MEA arising from the surface hyphae, hyaline aerial mycelium; *stipes* determinate, more frequently indeterminate, verrucolose, septate, in some cases arising from peg foot or initials suggesting foot-cells (Figure 6c–f); terminal penicilli more frequently terverticillate, and in few cases quaterverticillate, or even more branched, symmetric, or asymmetric, *rami* (first branch) larger than metula and phialides, 10.5–16.7 µm × 2.5–4 µm; metulae in well-defined verticils of 2–4, 7.8–9.7 µm × 1–3 µm, verrucolose (Figure 6d). Conidiogenous cells phialides, 6.0–9.4 × 1.9–2.9 µm, 3–4 per metula, typically cylindroidal without distinct neck, walls verruculose (Figure 6e); conidia on substrate very abundant, ellipsoidal or clavate, mostly 2.7–3.2 × 1.3–2.3 µm; conidial chains around 60–80 µm in length, in well defined, persistent, parallel columns (Figure 6f).

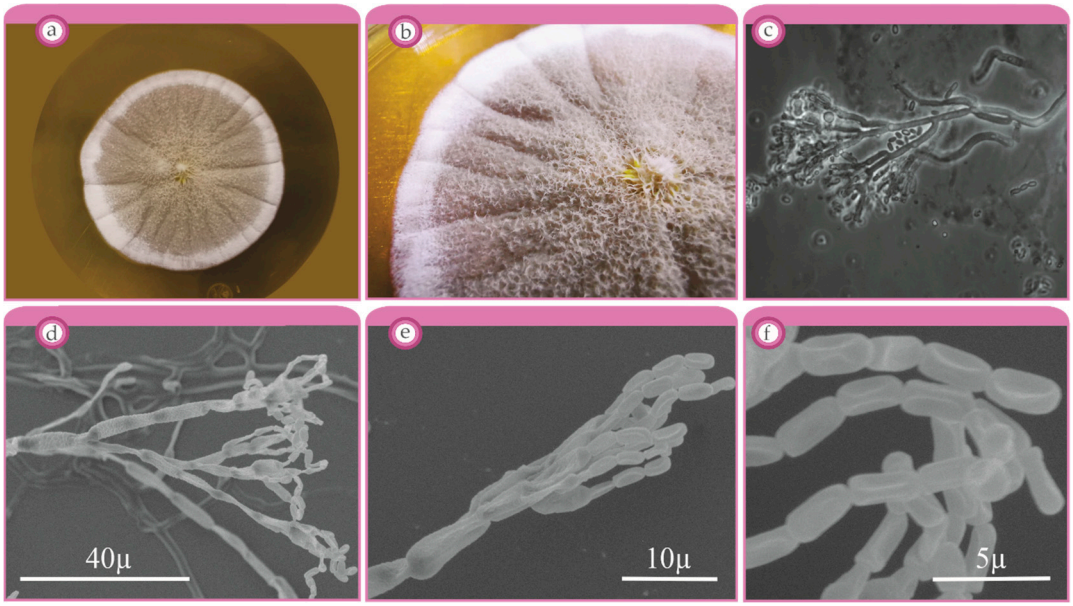


**Figure 6.** Colonies and microscopy characteristics of the strains obtained from *J. flaccida-P. deleoni* and clustered with *G. langdonii-Geosmithia* sp. 32 clade. (**a**,**b**) Colonial morphology in MEA; (**c**) conidia at 14 days of incubation in MEA using blue cotton in phases microscopy (40X); (**d**–**f**) electronic microscopy of penicilli and conidia.

On MEA, at 25 °C, 14 d: Colonies 45–70 mm diameter, radially furrowed, center low or slightly raised, surface texture velutinous, with masses of penicilli forming crust of conidia to 500 µm deep, or in some areas overlaid by an aerial mycelium also bearing penicilli, or almost floccose without a crustose pattern in some strain; margins narrow or lobate, submerged (to 5 mm broad); aerial and substrate mycelium hyaline or pale yellow, rusty or olivaceous; substrate mycelium sparse, not forming tough basal felt; heavy conidiogenesis, uncolored to pale in older areas; exudate absent or clear and uncolored; soluble pigment absent; reverse pale yellow, amber yellow to rusty (Figure 6a,b).

On CZA, 25 °C, 7 d: Colonies 25 mm diameter, 14 d: Colonies 50 mm diameter, sporulation low and white, other similar to MEA, soluble pigment absent; in this media did not show pigment.

On PAM, 25 °C, 7 d: Colonies 30 mm diameter, 14 d: Colonies 65 mm diameter, sporulation low and white, other similar to MEA, soluble pigment absent; in this media did not show pigment.

On MEA, 37 °C, 14 d. No growth.

Strains obtained from *J. flaccida-P. deleoni* (Figure 7a–f). Conidiophores on MEA arising from the surface hyphae, hyaline aerial mycelium; *stipes* indeterminate erect and less frequently determinate, conspicuously verrucose, septate, arising from peg foot or initials suggesting foot-cells (Figure 7c–e); penicilli terminal terverticillate (more frequently), quaterverticillate, or even more branched often

asymmetric, *rami* (first branch) larger than metula and phlialides, often 12.8–16.3 µm × 2.8–4.5 µm; metulae in well-defined verticils of 3–5, 6.6–8.3 µm × 1.8–2.7 µm, clearly verrucose (Figure 7d,e). Conidiogenous cells phialides, 6.9–8.9 µm × 2.0–2.6 µm, 3–5 per metula, typically cylindrical, walls verruculose, slightly proliferating (Figure 7d,e). Conidia on substrate mycelium very abundant, oval, in chains truncate basally, mostly 3.6–4.4 × 1.8–2.5 µm; conidial aerial chains around 450 µm in length, in well defined, longer conidial chains tangled (Figure 7d,f).

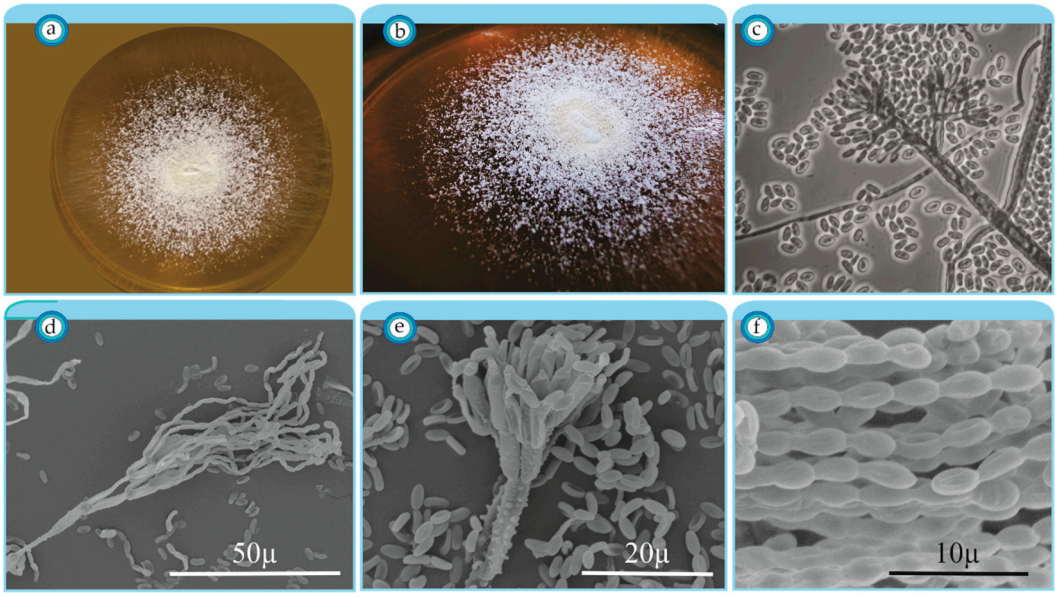


**Figure 7.** Colonies and microscopy characteristics of strains obtained from *J. flaccida-P. deleoni* and clustered with *Geosmithia* sp. 21-*G. xerotolerans* clade. (**a**,**b**) Colonial morphology in MEA; (**c**) conidia at 14 days of incubation in MEA using blue cotton in phases microscopy (40×); (**d**–**f**) electronic microscopy of penicilli and conidia.

On CZA, 25 °C, 7 d: Colonies 20 mm diameter, 14 d: Colonies 53 mm diameter, sporulation low and white, other similar to MEA, soluble pigment absent; in this media did not show pigment.

On PAM, 25 °C, 7 d: Colonies 30 mm diameter, 14 d: Colonies 65 mm diameter, sporulation low and white, other similar to MEA, soluble pigment absent; in this media did not show pigment.

On MEA, 37 °C, 14 d. No growth.

## 4. Discussion

In the present study, a systematic sampling using branch sections of Juniper species as a lure for bark beetles of the genus *Phloeosinus* allowed us to explore the diversity of *Geosmithia* fungal species in Nuevo León state, center of Sierra Madre Oriental (SMO), northeast Mexico. This is the first study that documents the symbiotic relationship of this fungus genus associated with its bark beetle vectors and host trees (vector galleries), in this country. Phylogenetic analysis based on internal transcribed spacer region (ITS) sequences supported the presence of strains associated with *Geosmithia langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*Geosmithia xerotolerans* clades in this geographical region. The characterization of colonies and conidiophores of these strains showed conspicuous morphological differences respect to those previously reported in the described species within their respective clades. Together, morphological and genetic differences found in Mexican *Geosmithia* suggest that both strains from Nuevo León could correspond to undescribed species in the genus.

### *4.1. Identity of Geosmithia Strains*

In most *Geosmithia* members, ITS allow species-level identification, as such, it has been used as a "DNA barcode" to document the diversity of this taxon in different geographical regions around the

world [29]. The recognition of monophyletic clusters using this marker, together with morphological attributes, is used as a criterion to recognize species in this genus [13,19,27,29]. In the present study, the molecular assignation (BLAST) and the ITS based phylogenies of 12 *Geosmithia* Nuevo León strains corresponded to two different lineages within this genus (Figures 3–5). Isolates collected in Galeana from the adult body of *Phloeosinus serratus* and its respective gallery system on *Juniperus coahuilensis* (Figure 2a,b) were clustered within *G. langdonii -Geosmithia*. sp. 32 clade (Figure 3); all strains collected in Iturbide from the adult body of *P. deleoni* and its gallery system on *J. flaccida* (Figure 2h,i) were clustered within the *Geosmithia* sp. 21-*G. xerotolerans* clade (Figure 3)

Phylogenetic analysis showed that sequences of both *Geosmithia* strains from Nuevo León, were monophyletic within their respective lineages ("*G. langdonii-Geosmithia* sp. 32" and "*Geosmithia* sp. 21-*G. xerotolerans*" clusters; Figures 4 and 5), and the average genetic distances among target sequences from Nuevo León concerning conspecific reference sequences within each group (*G. langdonii-Geosmithia* sp. 32 until up to 3.1%; *Geosmithia* sp. 21-*Geosmithia xerotolerans* until up to 5.1%) were higher than those calculated among conspecific reference sequences and other closeness *Geosmithia* members previously reported in GenBank. The monophyletic group within of *G. langdonii-Geosmithia* sp. 32 clade from Galeana displayed 2.0% of divergence than to the closer sequence of *G. langdonii* (HF546250.1; strain U91) from the *Phloeosinus thujae* vector and *Thuja occidentalis* host from California, USA (Figure 4). The monophyletic group within of *Geosmithia* sp. 21-*G. xerotolerans* clade from Iturbide displayed a 3.8% divergence and was closer to *Geosmithia* sp. 21 (AM421053.1; strain MK592) from *Hypoborus ficus* vector on *Ficus carica* host from Aquitánie, France ([19,29]; Figure 5).

The genetic differences observed among target sequences from Nuevo León within both clades and those previously reported are similar to the 2.2% divergence in the ITS sequence data displayed among other *Geosmithia* phylogenetic species and higher than 1.2% divergence in other species in the related genus *Penicillium* Link [55].

Of genetic distances in *G. langdonii-Geosmithia* sp. 32 clade, morphological differences in our isolate were found with respect to those displayed in the original description of *G. langdonii* [14]. Our isolated from Nuevo León has fewer phialides per metula (3–4), shorter phialides (6–9.5 µm), smaller conidia (2.7–2.3 µm length × 1.3–2.3 µm width), and shorter conidial chains (60–80 µm) with respect to *typus* from the Czech Republic that presented 3–15 phialides per metula; length of phialides 9–12 µm, size of conidia 4-5 × 2–2.5 µm, and length of conidial chains of 500 µm. Of these conspicuous differences, the conidiophore morphology was verriculose and we did not observe conidiophore verrucose as described previously [14]. Of genetic distances in *Geosmithia* sp. 21-*G. xerotolerans* clade, morphological differences in our isolate were found with respect to those displayed in the original description of *G. xerotolerans* [25]. Our isolated from Nuevo León showed shorter rami (12.8–16.3 × 2.8–4.5), higher number of metula per verticili (3–5), shorter metula up to 8.3 µm, shorter phialides (6.6–8.9 × 2.0–2.6 µm), bigger conidial chains (60–80 conidia) with respect to *typus* from Spain that presented rami of 7–15 × 2 µm; 2–3 metula per verticili, metula 7–15 × 2 µm, phialides 8–10 × 1.5–2 µm, conidial chains up to 20 conidia. Of these differences, the conidiophore morphology was clearly verrucose mycelium, septate, hyaline, and in contrast we did not observe a smooth wall.

The genetic and morphological differences found in our strains with respect to those previously described in the species from both clades *Geosmithia langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*G. xerotolerans* suggest that both *Geosmithia* lineages from Nuevo León could correspond to undescribed species in the genus; however, these results should be taken with caution. In the case of the clade *G. langdonii-Geosmithia* sp. 32 is necessary because the species included in it are indistinguishable using the ITS, and they can only be identified using other molecular markers such as TEF1 or TUB2 [19]. In the case of our strains clustered with *Geosmithia* sp. 21-*G. xerotolerans*, a more comprehensive morphological comparison was not possible because the *Geosmithia* sp. 21 has not been formally described or assigned to other of *Geosmithia* species yet; our phylogenetic analysis suggest that previous strains *Geosmithia* sp. 21 most probably can correspond to *Geosmithia xerotolerans* [25]. This species

was recently described based on morphological and molecular information, however the phylogenetic analysis that supported its description did not include molecular data of *Geosmithia* sp. 21, and thus did not consider the relatedness between these species. More iterative taxonomy studies need to be done including more molecular markers and isolated from other localities to evaluate the genetic and morphological variation of *Geosmithia* Mexican strains and its closer species to determinate the status of Mexican strains.

### *4.2. Geographic, Bark Beetle Vector, and Host Tree Records*

Several diversity studies have recorded new localities, vectors, and host species associated with strains of *G. langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*G. xerotolerans* clades, which led to them being recognized as generalist fungal [19], because they inhabit Palearctic and Nearctic regions and have been isolated from bark or ambrosia beetles (adults and galleries) as well endophyte on the same tree in a wide geographical range [14,15,19,29]. In the case of *G. xerotolerans*, it has only been recovered from the surface of a darkened house wall taken in Els Pallaresos, Tarragona province, Spain [25].

Our study extends the presence of these globally distributed clades in North America and provides the first records of this genus in Mexico (Figure 4). The new records of *Geosmithia* from Nuevo León, Mexico indicates that the distribution of both clades in America is substantially wider than previously reported, running through the west side of the Rocky Mountains (California, Colorado states), southeast of the USA (Florida, only *Geosmithia* sp. 21) to the North of Sierra Madre Oriental, Mexico. These records, together with those outside of America, support the distribution of *G. langdonii-Geosmithia* sp. 32 clade in temperate sub-Mediterranean (Slovakia, Czech Republic, and Bulgaria) and Mediterranean Europe (Portugal, Turkey; [15]), as well as from the western states of the USA (California, Colorado states; Kolarik et al., [19] and Northeast, Mexico; in *Geosmithia* sp. 21-*G. xerotolerans* clade, in temperate sub-Mediterranean and Mediterranean Europe (Azerbaijan, Croatia, France, Israel, Jordan, Italy, Slovenia, Spain, Syria, and Turkey), as well as in the western states of the USA (California, Colorado states) and southeastern (Florida [28] and Northeast, Mexico).

Strains of *G. langdonii*, *Geosmithia* sp. 32 and *Geosmithia* sp. 21 have been isolated from different families of Coleoptera vectors frequently associated with Scolytinae bark beetles [15,17,19]. Their specificity patterns and those of other conspecifics are congruent across different geographical regions, displaying a regular association with phloem-feeding bark beetles in a wide host spectrum [15,56]. Our *Geosmithia* strains correspond to this general pattern because both *G. langdonii* and *Geosmithia* sp. 21 were associated with the phloephagous bark beetle species, *Phloeosinus deleoni* and *P. serratus*, respectively, constituting new records of vector species. Including those vectors species recorded previously, strains from *G. langdonii-Geosmithia* sp. 32 clade have been isolated from at least 17 species of beetles corresponding to three families (Bostrichidae, Cerambicidae, and Curculionidae), from which 15 are Scolytids (Supplementary Table S1): Strains from *Geosmithia* sp. 21-*G. xerotolerans* clade have been isolated from at least 25 beetle species corresponding to three families (Bostrichidae, Cerambicidae, and Curculionidae), most of them Scolytinae (Supplementary Table S1).

The wide spectrum of vector species of *G. langdonii*, *Geosmithia* sp. 32 and *Geosmithia* sp. 21 is coupled with a high diversity of host plants corresponding to different families [19,29]. The Mexican strains from *Juniperus coahuilensis* and *J. flaccida* also increase the host species recorded of fungal species in both clades. In the strains from *G. langdonii-Geosmithia* sp. 32 clade, the host spectrum quantified at least 17 species through its geographical distribution (Supplementary Table S1), classified within seven plant families (Anacardiaceae, Asteraceae, Cupressaceae, Euforbeaceae, Fagaceae, Pinaceae, and Ulmacea). The strains from *Geosmithia* sp. 21-*G. xerotolerans* clade have been recorded from at least 17 host species, classified within five families (Cupressaceae, Fabaceae, Moraceae, Rosaceae, Oleaceae, and Pinaceae).

### 4.3. Geosmithia Diversity

The community structure of *Geosmithia* species in landscapes is driven principally by the diversity of both bark beetles and their host plant as well as their interactions [19,29]. On small ecological scales, previous data have supported that neighboring populations of the same vector species can transmit relative similar *Geosmithia* assemblages in the same or different host species [18]. As mentioned above, both sampling areas (Galeana and Iturbide municipalities) are in the north of the physiographic province Sierra Madre Oriental. Thus, they present similar environmental conditions, landscapes, climate, and seasonal rain regimes [19,29]. Given these common characteristics, their geographic proximity, and because in both areas, only one dominant arboreal species was found (*J. coahuilensis* and *J. flaccida*, respectively), each one associated with a unique bark beetle species (*P. serratus* and *P. deleoni*), a similar *Geosmithia* species composition pattern between them and low diversity in each were expected.

Our sampling, with multiple repetitions of cut branches as a lure of bark beetles, supports a low diversity of *Geosmithia*, with only one fungal species per geographical area, as reported by Kolarik [16,19], strains associated with G. *xerotolerans-Geosmithia* sp. 21 clade from "Iturbide" and strains associated with *G. langdonii-Geosmithia* sp. 32 clade from "Galeana", which indicates that the genus *Phloeosinus* harbors a low diversity of fungi, as was observed in other members of genus [16,19]. Both fungal species were recovered across multiple sampling sites in several tree branches and gallery systems (adults and tunnels) supporting a non-incidental association.

### 4.4. Entomochory in Geosmithia Strains

Despite that dispersion of *Geosmithia* species can be performed by different mediums as wind or water, the establishment of its communities has been explained by the vertical dispersion with vector insects [14–17,22,29,57] because species are isolated from the adult body and gallery systems. *Geosmithia* species from Nuevo León were isolated from the insect surface and its respective galleries. Particularly, conidia were located in pupal chambers (Figure 2), sites where metamorphosis occurs and the adults have direct contact with the spores, just before their emergence, which could promote a more efficient transmission and ensures horizontal transfer. To support this hypothesis, we found that 100% of the beetles and gallery samples of both bark beetle species in Nuevo León were coupled with *Geosmithia*; however, more studies are necessary to analyze the fungal growth within gallery systems and its role in beetle dispersion.

Although we sampledsome tree branches in each region, both fungal species were not found to co-exist, and each region presented a unique *Geosmithia* "species", associated with a particular plant composition and vector species; the sampling area at Galeana corresponded to semi-arid xerophytic scrub dominated by the *J. coahuilensis* species; in Iturbide, vegetation corresponded to semi-arid pine forest dominated by *J. flaccida*. These results indicate that alpha diversity in *Geosmithia* communities is low in small geographical scales that present few potential vectors and hosts, but that beta diversity is higher between landscapes that display different and particular species composition of hosts and vectors.

Even though the *Geosmithia* species developed stable symbiotic relationships with different bark beetle species and resemble ophiostomatoid fungi in their host and vector affinities and life strategy evolution, the ecological role of *Geosmithia* species in beetle galleries is unclear. Some recent studies suggest that the frequency of isolation of *Geosmithia* in *Phloeosinus* species indicates a closer symbiotic relationship among them. *Phloeosinus* Chapuis is constituted by more than 60 taxonomically valid species, 29 of them live on the American continent [31], of which 10 out of 29 (≈35%) had been sampled to search *Geosmithia*, displaying an incidence of 100% with almost one *Geosmithia* member isolated per bark beetle species [19,29]; such is the case of *P. cupressi, P. sequiae, P. canadensis,* and *P. punctatus* in which the same *Geosmithia* sp. 21 and *G. langdonii* were isolated, the last only form *P. cupressi* and *P. sequoiae*.

## 5. Conclusions

Our results document the presence of strains from *Geosmithia langdonii-Geosmithia* sp., 32 and *Geosmithia* sp. 21-*G xerotolerans* clades in Mexico, supporting their distribution in North America from the Rocky Mountains, as well as southeastern sections of the USA (only *Geosmithia* sp. 21) to North of Sierra Madre Oriental, Mexico. In North Mexico, these fungal strains were associated with the phloem-feeding bark beetle vectors *Phloeosinus serratus* and *P. deleoni*, and showed the capacity of developing in the gallery systems of insects on the host species *Juniperus coahuilensis* and *Juniperus flaccida*, respectively. Each fungal strain inhabits a particular forest community and displays a specific association with vector insects and host plants. Genetic and morphological data suggest that both Mexican *Geosmithia* strains correspond to potential new species.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4907/11/11/1142/s1, Table S1: Hosts and Scolytinae vectors species associated with strains from *Geosmithia langdonii-Geosmithia* sp. 32 and *Geosmithia* sp. 21-*G. xerotolerans* clades.

**Author Contributions:** Conceptualization, H.-G.J.A. and A.-T.F.; methodology, H.-G.J.A., C.-R.G., A.-O.N.G. and A.-T.F.; formal analysis, H.-G.J.A. and A.-T.F.; writing—original draft preparation, H.-G.J.A., C.-R.G. and A.-T.F.; writing—review and editing, H.-G.J.A., C.-R.G., H.-R.C., V.-T.L. and A.-T.F.; visualization, H.-G.J.A., A.-O.N.G. and A.-T.F.; supervision, C.-R.G. and A.-T.F.; funding acquisition, C.-R.G., H.-R.C., V.-T.L. and A.-T.F. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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